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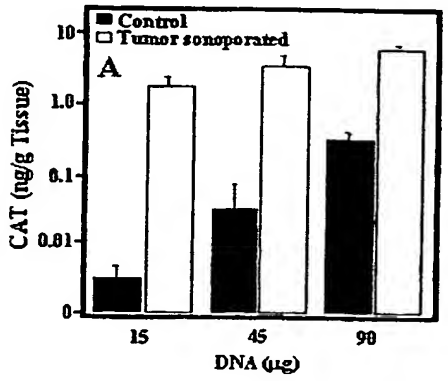
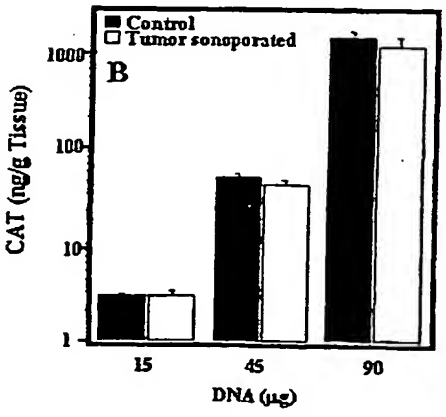
(54) Title: SONOPORATION OF TUMORS

(57) Abstract: The present invention relates to products and methods useful for delivering one or more nucleic acid molecules to a tumor and sonoporating the tumor. The products and methods permit preferential delivery and expression of the nucleic acid molecules in cells located in a tumor subject to sonoporation relative to expression in non-sonoporated tissues.





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SONOPORATION OF TUMORS

Introduction

The present invention relates to products and methods useful for delivering one or more nucleic acid molecules to a tumor and sonoporating the tumor. This application is a continuation in part of, and claims priority from, US Application Serial Number 60/ 146,072 filed July 28, 1999.

Background of the Invention

The following information is presented solely to assist the understanding of the reader, and none of the information is admitted to describe or constitute prior art to the claims of the present invention.

In the past, non -viral administration of nucleic acids *in vivo* has been pursued by a variety of methods. These include lipofectin/liposome fusion: Felgner *et al., Proc. Natl. Acad. Sci.*, Volume 84, pp. 7413 - 7417 (1987); and transferrin:transferrin receptor delivery of nucleic acid to cells: Wagner *et al., Proc. Natl. Acad. Sci.*, Volume 87, pp. 3410 - 3414 (1990). The use of a specific composition consisting of polyacrylic acid has been disclosed in International Patent Publication No. WO 94/24983. Naked DNA has been administered as disclosed in International Patent Publication No. WO 90/11092.

Gene therapy has quickly become a major area of research in drug development. A key technological barrier to commercialization of gene therapy, however, is the need for practical and effective gene delivery methods. The primary problem of gene injection by conventional needle-syringe methods is that genetic material must be injected in large quantities into the target site because of the inefficiency of attempting to diffuse genetic material into the cells' nuclei and the need to overwhelm enzyme systems that immediately move to destroy the injected nucleic acid molecules. Therapeutic injection technology using a needle-syringe has progressed relatively slowly.

Gene transfer strategies targeting tumor endothelium to provide sustained, high, and local concentrations of anti-angiogenesis mediators, immunocytokines, or cytotoxic proteins thus minimizing systemic toxicity have potential therapeutic value. Current gene delivery systems transfect cells *in vivo* in a manner largely determined by blood flow and site of introduction (Mulligan, R.C. (1993), Science 260, 926-932; Felgner, P.L. and Rhodes, G.

(1991), Nature 349, 351-352). Because of this, the ability of these systems to deliver therapeutic genes to target cells *in vivo* is limited.

For example, cationic liposomes have been widely used for gene transfer into endothelial cells *in vivo* (Brigham, K.B., et al. (1989), Am. J. Med. Sci. 298, 278 – 281; Hofland, H.E.J., et al. (1997), Pharm. Res. 14, 742 – 749; Liu, F., et al. (1997), Gene Therapy 4, 517 – 523; Mahato, R.I., et al. (1998), Hum. Gene Ther. 9, 2083 – 2099; Rolland, A.P. (1998), Critical Reviews in Therapeutic Drug Carrier Systems 15, 143 - 198). The utility of current cationic liposome-based systems for targeting tumor endothelium is limited due to lack of target cell specificity and low *in vivo* gene transfer efficiency (Lesoon - Wood, L.A., et al. (1995), Hum. Gene. Ther. 6, 395 – 405; Anwer et al. (Human gene Therapy, submitted)).

Modification of liposome surface by covalent conjugation of monoclonal antibodies or another targeting moieties (e.g., specific peptides and lipids) has been proposed to improve tumor-specific gene delivery (Boulikas, T. (1996), Int. J. Oncol. 9, 941 – 954; Kong, H.L., and Crystal, R.G. (1998), J. Natl. Cancer Inst. 90, 273 – 286; Pietersz, G.A. and McKenzie, I.F.C. (1992), Immunol. Rev. 129, 57 – 80; Thorpe, P.E. and Derbyshire, E.J. (1997), J. Cont. Release 48, 277 - 288; Kircheis, R., et al. (1997), Gene Therapy 4, 409 - 418). Mechanical methods such as electroporation and jet injections have also been described as useful external means to enhance gene transfer in target tissue (Gallo, S.A., et al. (1997), Biophys. J. 72, 2805-2811).

Ultrasound-mediated delivery has potential as a powerful new method for enhancing and targeting administration of therapeutic compounds into and across cells and tissues. Ultrasound-enhanced delivery to cells has been demonstrated *in vitro* by uptake of extracellular fluid, drugs, and DNA into cells (Liu, J., et al. (1998), Pharm. Res. 15, 918-924; Mitragotri, et al. (1996), Pharm. Res. 13, 411-420; Wyber, J.A., et al. (1997), Pharm. Res. 14, 750-756; Tata, D.B., et al. (1997), Biochem. Biophys. Res. Commun. 234, 64-67).

Despite these recent advances there remains need for additional and improved sonoporation related products and methods.

Summary of the Invention

This invention features compositions and methods for enhancing the administration to and uptake of nucleic acids in an organism. The data presented herein demonstrates that ultrasound delivery of formulated nucleic acid molecules is a more favorable method for nucleic acid delivery to tumors when compared with non-ultrasound delivery methods. As used herein, sonoporation is used to describe use of ultrasound treatment to facilitate the transfection of cells with formulations including nucleic acids. The term sonoporation is used to mean ultrasound treatment without any limitation to specific mechanisms of cellular changes that may be induced by the treatment.

The invention provides a method to deliver nucleic acid molecules formulated with an agent that facilitates transfection (preferably a cationic lipid or PINC™ agent as described below) to an organism by using an apparatus configured and arranged to administer molecules by applying ultrasound waves to the cells of an organism. Thus, the present invention allows for superior delivery of nucleic acid molecules into cells (preferably tumor cells) *in vivo* by the combination of an ultrasound device and formulated nucleic acid molecules. Furthermore, the present invention also allows for treatment of diseases and vaccination, especially with respect to various cancers.

An ultrasound method is described that provides for specific targeting of gene transfer into primary tumors, preferably after systemic administration of cationic lipid/plasmid complexes. Intravenous administration of N-[(1-(2,3-dioleyloxy) propyl)]-N-N-N-trimethylammonium chloride (DOTMA)-based transfection complexes into tail vein of subcutaneous squamous cell tumor bearing mice led to plasmid uptake and reporter gene expression in tumor lesions. Expression was also observed in non-tumor tissues including lung and liver. Application of ultrasound to tumor lesions after i.v. administration of lipid/plasmid complexes enhanced reporter gene expression in tumor by 6 - 270 fold. The enhancement in gene expression was treatment site specific since no increase was observed in non-tumor tissue.

Several parameters including time of ultrasound or sonoporation treatment, interval between plasmid injection and sonoporation, and DNA dose influenced the outcome of sonoporation. The increase in reporter gene expression by sonoporation at 15, 45, and 90 microgram plasmid dose was 270%, 40%, and 6% over control. Quantitative PCR showed 3-fold increase in tumor uptake of DNA after sonoporation with no increase in DNA uptake by lung. Fluorescent microscopy showed that sonoporation treatment promoted DNA plasmid

uptake by endothelial cells of tumor and increased perinuclear localization of the sonoporated DNA.

Local sonoporation over a tumor in conjunction with systemic administration of an IL-12 encoding plasmid resulted in preferential expression of IL-12 in the tumor relative to tissue not subject to sonoporation. Enhanced expression of IL-12 in the local tumor area as a result of sonoporation resulted in significant reduction in tumor size compared with administration of the IL-12 gene without sonoporation.

These data demonstrate the application of sonoporation as an effective method for targeting systemically administered genes to tumor endothelium for cancer gene therapy.

The present invention features an ultrasound method that provides for specific enhancement in gene transfer to tumor lesions transfected by systemic, subcutaneous or intratumoral administration of transfection facilitating agent/DNA complexes. The enhancement in gene transfer was restricted to the local tumor treatment site since no effects were observed in tissues distal to the site of ultrasound administration. Therefore, ultrasound treatment may prove to be a useful method to enhance systemic gene delivery into tumor without affecting non-target tissue.

In a first aspect, the invention provides a method for delivering a nucleic acid molecule to a tumor, preferably a primary tumor, comprising the steps of systemically administering a transfection facilitating agent/plasmid complex preferably a liposome/plasmid complex, to the tumor and sonoporating the tumor. Sonoporation typically follows administration of the gene, but sonoporation prior to (preferably immediately prior to) administration also results in enhancement of gene delivery and expression.

By "delivery" or "delivering" is meant transportation of nucleic acid molecules to desired cells or any cells. The nucleic acid molecules may be delivered to multiple cell lines, including the desired target. Delivery results in the nucleic acid molecules coming in contact with the cell surface, cell membrane, cell endosome, within the cell membrane, nucleus or within the nucleus, or any other desired area of the cell from which transfection can occur within a variety of cell lines which can include but are not limited to; tumor cells, epithelial cells, Langerhan cells, Langhans cells, littoral cells, keratinocytes, dendritic cells, macrophage cells, Kupffer cells, muscle cells and lymphocytes. Preferably, the formulation is delivered to the cells by sonoporation and the nucleic acid molecule component is not significantly sheared upon delivery, nor is cell viability directly effected by the sonoporation process.

The term "nucleic acid" as used herein refers to both RNA and DNA including: cDNA, genomic DNA, plasmid DNA or condensed nucleic acid, nucleic acid formulated with cationic lipids, nucleic acid formulated with peptides, antisense molecules, cationic substances, RNA or mRNA. In a preferred embodiment, the nucleic acid administered is plasmid DNA that includes a "vector". The nucleic acid can be, but is not limited to, a plasmid DNA vector with a eukaryotic promoter which expresses a protein with potential therapeutic action, such as, for example; hGH, VEGF, EPO, IGF-1, IPO, Factor IX, IFN-alpha, IFN-beta, IL-2, IL-12, or the like.

As used herein, the term a "plasmid" refers to a construct made up of genetic material (i.e., nucleic acids). It includes genetic elements arranged such that an inserted coding sequence can be transcribed in eukaryotic cells. Also, while the plasmid may include a sequence from a viral nucleic acid, such viral sequence preferably does not cause the incorporation of the plasmid into a viral particle, and the plasmid is therefore a non-viral vector. Preferably a plasmid is a closed circular DNA molecule.

The term "vector" as used herein refers to a construction comprised of genetic material designed to direct transformation of a targeted cell. A vector contains multiple genetic material, preferably contiguous fragments of DNA or RNA, positionally and sequentially oriented with other necessary elements such that the nucleic acid can be transcribed and when necessary translated in the transfected cells.

The term "transfection facilitating agent" as used herein refers to an agent that forms a complex with the nucleic acid. This molecular complex is associated with nucleic acid molecule in either a covalent or a non-covalent manner. The transfection facilitating agent should be capable of transporting nucleic acid molecules in a stable state and of releasing the bound nucleic acid molecules into the cellular interior. DNA extraction methods, methods of immunofluorescence, or well-known reporter gene methods such as for example CAT, or LacZ containing plasmids, could be used in order to determine the transfection efficiency. The transfection facilitating agent should also be capable of being associated with nucleic acid molecules and may be lyophilized or freeze dried and rehydrated prior to delivery and sonoporation.

In addition, the transfection facilitating agent may prevent lysosomal degradation of the nucleic acid molecules by endosomal lysis. Furthermore, the transfection facilitating agent may allow for efficient transport of the nucleic acid molecule through the cytoplasm of the cell to the nuclear membrane and into the nucleus and provide protection.

In one embodiment transfection facilitating agents are non-condensing polymers, oils and surfactants. Non- condensing polymers have been found to be particularly suitable for injection into the site of desired expression such as in intra-tumoral administration. These may be suitable for use as compounds which prolong the localized bioavailability of a nucleic acid: polyvinylpyrrolidones; polyvinylalcohols; propylene glycols; polyethylene glycols; polyvinylacetates; poloxamers (Pluronics)(block copolymers of propylene oxide and ethylene oxide, relative amounts of the two subunits may vary in different poloxamers); poloxamines (Tetronics); ethylene vinyl acetates; celluloses, including salts of carboxymethylcelluloses, methylcelluloses, hydroxypropyl-celluloses, hydroxypropylmethylcelluloses; salts of hyaluronates; salts of alginates; heteropolysaccharides (pectins); phosphatidylcholines (lecithins); miglyols; polylactic acid; polyhydroxybutyric acid. Some of these compounds may be used as, and are considered protective, interactive, non-condensing compounds (PINC) and others as sustained release compounds, while some may be used in either manner under the respectively appropriate conditions.

In another embodiment, cationic condensing agents such as cationic lipids, peptides, or lipopetides, or for example, dextrans, chitosans, dendrimers, polyethyleneimine (PEI), or polylysine, may associate with the nucleic acid molecule and may facilitate transfection in conjunction with sonoporation.

The PINC enhances the delivery of the nucleic acid molecule to mammalian cells *in vivo*, and preferably the nucleic acid molecule includes a coding sequence for a gene product to be expressed in the cell. In particular, PINC has been found useful for direct injection into muscle, tumors or organs. In many cases, the relevant gene product is a polypeptide or protein. The PINC may be used under conditions so that the PINC does not form a gel, or so that no gel form is present at the time of administration at about 30-40°C. Thus, in these compositions, the PINC is present at a concentration of 30% (w/v) or less. In certain embodiments, the PINC concentration is still less, for example, 20% or less, 10% or less, 5% or less, or 1% or less. Thus, these compositions differ in compound concentration and functional effect from uses of these or similar compounds in which the compounds are used at higher concentrations, for example in the ethylene glycol mediated transfection of plant protoplasts, or the formation of gels for drug or nucleic acid delivery. In general, the PINCs are not in gel form in the conditions in which they are used as PINCs, though certain of the compounds may form gels under some conditions.

In connection with the protective, interactive, non-condensing compounds for these compositions, the term "non-condensing" means that an associated nucleic acid is not condensed or collapsed by the interaction with the PINC at the concentrations used in the compositions. Thus, the PINCs differ in type and/or concentration from such condensing polymers. Examples of commonly used condensing polymers include polylysine, and cascade polymers (spherical polycations).

The term "protects" or "protective" or "protected" as used herein refers to an effect of the interaction between such a compound and a nucleic acid such that the rate of degradation of the nucleic acid is decreased in a particular environment, thereby prolonging the localized bioavailability of the nucleic acid molecule. Such degradation may be due to a variety of different factors, which specifically include the enzymatic action of a nuclease. The protective action may be provided in different ways, for example, by exclusion of the nuclease molecules or by exclusion of water.

The term "interactive" as used herein refers to the interaction between PINC's and nucleic acid molecules and/or cell wall components. Preferably, PINC polymers are capable of directly interacting with moieties of nucleic acid molecules and/or cell wall components. These interactions can facilitate transfection by, for example, helping associate the nucleic acid molecule-PINC complex closely with the cell wall as a result of biochemical interactions between the PINC and the cell wall and thereby mediate transfection. These interactions may also provide protection from nucleases by closely associating with the nucleic acid molecule.

Also in connection with such compounds and an associated nucleic acid molecule, the term "enhances the delivery" means that at least in conditions such that the amounts of PINC and nucleic acid is optimized, a greater biological effect is obtained than with the delivery of nucleic acid in saline. Thus, in cases where the expression of a gene product encoded by the nucleic acid is desired, the level of expression obtained with the PINC:nucleic acid composition is greater than the expression obtained with the same quantity of nucleic acid in saline for delivery by a method appropriate for the particular PINC/coding sequence combination.

In preferred embodiments of the above compositions, the PINC is polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), a PVP-PVA co-polymer, N-methyl-2-pyrrolidone (NM2P), ethylene glycol, or propylene glycol. In compositions in which a

Poloxamer (Pluronics) is used, the nucleic acid is preferably not a viral vector, i.e., the nucleic acid is a non-viral vector.

In other preferred embodiments, the PINC is bound with a targeting ligand. Such targeting ligands can be of a variety of different types, including but not limited to galactosyl residues, fucosal residues, mannosyl residues, carntitine derivatives, monoclonal antibodies, polyclonal antibodies, peptide ligands, and DNA-binding proteins. The targeting ligands may bind with receptors on cells such as antigen-presenting cells, hepatocytes, myocytes, epithelial cells, endothelial cells, and cancer cells.

In connection with the association of a targeting ligand and a PINC, the term "bound with" means that the parts have an interaction with each other such that the physical association is thermodynamically favored, representing at least a local minimum in the free energy function for that association. Such interaction may involve covalent binding, or non-covalent interactions such as ionic, hydrogen bonding, van der Waals interactions, hydrophobic interactions, and combinations of such interactions.

While the targeting ligand may be of various types, in one embodiment the ligand is an antibody. Both monoclonal antibodies and polyclonal antibodies may be utilized.

The nucleic acid may also be present in various forms. Preferably the nucleic acid is not associated with a compounds(s) that alter the physical form, however, in other embodiments the nucleic acid is condensed (such as with a condensing polymer), formulated with cationic lipids, formulated with peptides, or formulated with cationic polymers.

In preferred embodiments, the protective, interactive non-condensing compound is polyvinyl pyrrolidone, and/or the plasmid is in a solution having between 0.5% and 50% PVP, more preferably about 5% PVP. The DNA preferably is at least about 80% supercoiled, more preferably at least about 90% supercoiled, and most preferably at least about 95% supercoiled.

The compounds which protect the nucleic acid and/or prolong the localized bioavailability of a nucleic acid may achieve one or more of the following effects, due to their physical, chemical or rheological properties: (1) protect nucleic acid, for example plasmid DNA, from nucleases due to steric, viscosity, or other effects such as shearing; (2) increase the area of contact between nucleic acid, such as plasmid DNA, through extracellular matrices and over cellular membranes, into which the nucleic acid is to be taken up; (3) concentrate nucleic acid, such as plasmid DNA, at cell surfaces due to water exclusion; (4) indirectly facilitate uptake of nucleic acid, such as plasmid DNA, by disrupting cellular membranes due to osmotic, hydrophobic or lytic effects; (5) indirectly facilitate uptake of nucleic acids by

allowing diffusion of protected nucleic acid chains through tissue at the administration site; and (6) indirectly facilitate uptake of nucleic acid molecules through pore, holes, openings in the cells formed as a result of the electroporation process.

By "prolonging the localized bioavailability of a nucleic acid" is meant that a nucleic acid administered to an organism in a composition comprising a transfection facilitating agent will be available for uptake by cells for a longer period of time than if administered in a composition without such a compound, for example when administered in a saline solution. This increased availability of nucleic acid to cells could occur, for example, due to increased duration of contact between the composition containing the nucleic acid and a cell or due to protection of the nucleic acid from attack by nucleases. The compounds that prolong the localized bioavailability of a nucleic acid are suitable for internal administration.

By "suitable for internal administration" is meant that the compounds are suitable to be administered within the tissue of an organism, for example within a muscle or within a joint space, epidermally, intradermally or subcutaneously. Properties making a compound suitable for internal administration can include, for example, the absence of a high level of toxicity to the organism as a whole.

The plasmid may also be complexed with a liposome formed from the one or more cationic lipids. Preferably the cationic lipid is DOTMA and the neutral co-lipid is cholesterol (chol). DOTMA is 1,2-di-O-octadecenyl-3-trimethylammonium propane, which is described and discussed in Eppstein et al., U.S. Patent 4,897,355, issued January 30, 1990, which is incorporated herein by reference. However, other lipids and lipid combinations may be used in other embodiments. A variety of such lipids are described in Gao & Huang, 1995, *Gene Therapy* 2:710-722, which is hereby incorporated by reference.

As the charge ratio of the cationic lipid and the DNA is also a significant factor, in preferred embodiments the DNA and the cationic lipid are present is such amounts that the negative to positive charge ratio is about 1:3. While preferable, it is not necessary that the ratio be 1:3. Thus, preferably the charge ratio for the compositions is between about 1:1 and 1:10, more preferably between about 1:2 and 1:5.

The term "cationic lipid" refers to a lipid which has a net positive charge at physiological pH, and preferably carries no negative charges at such pH. An example of such a lipid is DOTMA. Similarly, "neutral co-lipid" refers to a lipid which has is usually uncharged at physiological pH. An example of such a lipid is cholesterol. Cationic lipid formulations have been found useful for systemic delivery.

Thus, "negative to positive charge ratio" for the DNA and cationic lipid refers to the ratio between the net negative charges on the DNA compared to the net positive charges on the cationic lipid.

As the form of the DNA affects the expression efficiency, the DNA preferably is at least about 80% supercoiled, more preferably at least about 90% supercoiled, and most preferably at least about 95% supercoiled. The composition preferably includes an isotonic carbohydrate solution, such as an isotonic carbohydrate solution that consists essentially of about 10% lactose. In preferred embodiments, the composition the cationic lipid and the neutral co-lipid are prepared as a liposome having an extrusion size of about 800 nanometers. Preferably the liposomes are prepared to have an average diameter of between about 20 and 800 nm, more preferably between about 50 and 400 nm, still more preferably between about 75 and 200 nm, and most preferably about 100 nm. Microfluidization is the preferred method of preparation of the liposomes.

The term "sonoporation device", as used herein relates to an apparatus that is capable of causing or causes uptake of nucleic acid molecules into the cells of an organism by ultrasound means. The cell membrane may thus destabilize and result in the formation of passageways or pores in the cell membrane. The type of sonoporation device is not considered a limiting aspect of the present invention. The primary importance of a sonoporation device is, in fact, the capability of the device to deliver formulated nucleic acid molecules into the cells of an organism.

The term "apparatus" as used herein relates to the set of components that upon combination allow the delivery of formulations of nucleic acid molecules and transfection facilitating agents into the cells of an organism by sonoporation delivery methods.

The term "skin" refers to the outer covering of a mammal consisting of epidermal and dermal tissue and appendages such as sweat ducts and hair follicles. Skin can comprise the hair of a mammal in cases where the mammal has an epidermis that is covered by hair. In mammals which have enough hair to be considered fur or a pelt it is preferable to shave the hair, leaving primarily skin.

The term "organism" as used herein refers to common usage by one of ordinary skill in the art. The organism can include; micro-organisms, such as yeast or bacteria, plants, birds, reptiles, fish or mammals. The organism can be a companion animal or a domestic animal. Preferably the organism is a mammal and is therefore any warm-blooded organism. More preferably the mammal is a human.

In another embodiment the method results in an immune response, preferably a humoral immune response targeted for the protein product encoded by the nucleic acid molecule, such as an antibody response. In other situations the immune response preferably is a cytotoxic T-lymphocyte response.

The term "immune response" as used herein refers to the mammalian natural defense mechanism that can occur when foreign material is internalized. The immune response can be a global immune response involving the immune system components in their entirety. Preferably the immune response results from the protein product encoded by the formulated nucleic acid molecule. The immune response can be, but is not limited to: antibody production, T-cell proliferation /differentiation, activation of cytotoxic T-lymphocytes, and/or activation of natural killer cells. Preferably the immune response is a humoral immune response. However, as noted above, in other situations the immune response, preferably, is a cytotoxic T-lymphocyte response.

The term "humoral immune response" refers to the production of antibodies in response to internalized foreign material. Preferably the foreign material is the protein product encoded by a formulated nucleic acid molecule internalized by injection with a needle free device.

In a preferred embodiment the method results in enhanced transfection of cells as a result of a better method for gene delivery, when compared to sonoporation of non-formulated (naked) nucleic acid. The enhanced transfection can be measured by transfection reporter methods commonly known in the art such as, for example, assays for CAT gene product activity, or LacZ gene product activity, and the like.

The "commercial package" or the "container" can include instructions furnished to allow one of ordinary skill in the art to make formulated nucleic acid molecules. The instructions may furnish steps to make the compounds used for formulating nucleic acid molecules. Additionally, the instructions may include methods for testing the formulated nucleic acid molecules that entail establishing if the formulated nucleic acid molecules are damaged upon injection after electroporation. The kit may also include notification of an FDA approved use and instructions.

The term "transfection" as used herein refers to the process of introducing DNA (e.g., formulated DNA expression vector) into a cell, thereby, allowing cellular transformation. Following entry into the cell, the transfected DNA may: (1) recombine with that of the host;

(2) replicate independently as a plasmid or temperate phage; or (3) be maintained as an episome without replication prior to elimination.

As used herein, "transformation" relates to transient or permanent changes in the characteristics (expressed phenotype) of a cell induced by the uptake of a vector by that cell. Genetic material is introduced into a cell in a form where it expresses a specific gene product or alters the expression or effect of endogenous gene products.

Transformation of the cell may be associated with production of a variety of gene products including protein and RNA. These products may function as intracellular or extracellular structural elements, ligands, hormones, neurotransmitters, growth regulating factors, enzymes, chemotaxins, serum proteins, receptors, carriers for small molecular weight compounds, drugs, immunomodulators, oncogenes, cytokines, tumor suppressors, toxins, tumor antigens, antigens, antisense inhibitors, triple strand forming inhibitors, ribozymes, or as a ligand recognizing specific structural determinants on cellular structures for the purpose of modifying their activity. This list is only an example and is not meant to be limiting.

Administration as used herein refers to the route of introducing the formulated nucleic acid molecules of the invention into the body of cells or organisms. Administration includes the use of sonoporation methods to targeted areas of the mammalian body such as tumors, the muscle cells and the lymphatic cells in regions such as the lymph nodes.

Prior to administration, the nucleic acid molecules of the invention can be formulated with at least one transfection facilitating agent type of molecule. For example, the molecular complexes can be formulated with cationic lipids or PINC's such as polyvinyl-pyrrolidone as described herein. Formulation techniques are provided herein by example.

The summary of the invention described above is not limiting and other features and advantages of the invention will be apparent from the following detailed description of the invention and from the claims.

Brief Description of The Drawings

Figure 1 shows the effect of ultrasound treatment on cationic liposome mediated systemic gene transfer in mouse s.c. SCCVII tumor and lung. CAT expression levels in tumor (A) and lung (B) were measured 18-20 hr after DNA administration.

Figure 2 shows the effect of ultrasound treatment on tumor/lung expression ratio after systemic administration of cationic lipid/plasmid complexes at different DNA doses.

Figure 3 shows the influence of sonoporation time on CAT expression in s.c. SCCVII tumors after tail vein administration of DOTMA:CHOL/plasmid complexes.

Figure 4 shows the effect of time interval between DNA administration and sonoporation on CAT expression in s.c. SCCVII tumors after tail vein administration of DOTMA:CHOL/plasmid complexes.

Figure 5 show the effect of sonoporation prior to DNA administration.

Figure 6 shows the relative sonoporation enhancement of plasmid DNA uptake by s.c. SCCVII tumors versus lung after tail vein administration of plasmid/ DOTMA:CHOL complexes.

Figure 7 shows the effect of ultrasound treatment on IL-12 expression in s.c. tumors by tail vein injection of IL-12 plasmid complexed with DOTMA:CHOL liposomes.

Figure 8 shows the effect of ultrasound treatment on inhibition of tumor growth following systemic administration of IL-12 transfection complexes

Detailed Description of the Preferred Embodiments

The delivery, preferably to tumors, of formulations of nucleic acid molecules and transfection facilitating agents by the use of sonoporation device represents a novel approach to gene delivery. The present invention offers a nucleic acid delivery apparatus that provides, for example, an increased number of transfected cells, and also an increased immune response when compared to previous methods as a direct result of providing a more efficient method for transforming cell lines and, thereby increase the production of therapeutic proteins or proteins that potentially trigger an immune response. The invention provides the advantage of allowing the uptake of formulated nucleic acid molecules by specifically targeted cells in vivo.

Delivery of formulated nucleic acid molecules by sonoporation methods results in the formulated nucleic acid molecules gaining access to the cellular interior more directly through the destabilization of the cell wall and/or by the formation of pores as a result of the sonoporation. Furthermore, in certain instances multiple cell types can be targeted, thus allowing contact to many more cell types than in conventional methods. Thus, the present invention provides an enhanced delivery of nucleic acid molecules and also provides a more efficient gene delivery system which can be used to generate an immune response, modulate aspects of the cell cycle or cell physiology, or provide a method to achieve other gene delivery related therapeutic methods such as anti-tumor therapy.

Sonoporation of formulated nucleic acid molecules to an organism, depends on several factors which are discussed below, including transfection efficiency and the composition of the formulated nucleic acid molecule.

Cationic lipid-based formulations have been widely described to achieve gene transfer into normal endothelial tissue by systemic administration (Brigham, K.B., et al. (1989), Am. J. Med. Sci. 298, 278 – 281; Hofland, H.E.J., et al. (1997), Pharm. Res. 14, 742 – 749; Liu, F., et al. (1997), Gene Therapy 4, 517 - 523; Mahato, R.I., et al. (1998), Hum. Gene Ther. 9, 2083 – 2099; Rolland, A.P. (1998), Critical Reviews in Therapeutic Drug Carrier Systems 15, 143 – 198; Lesoon-Wood, L.A., et al. (1995), Hum. Gene. Ther. 6, 395 - 405). The present application describes an ultrasound treatment method that provides for specific increase in gene transfer into mouse primary tumors transfected by systemic administration of cationic lipid/plasmid complexes. The sonoporation treatment did not affect gene expression in non-tumor tissues and did not produce adverse effects on the animals as assessed by gross tissue examination and general physical health of the animals.

Ultrasound has been a well established diagnostic and therapeutic tool in medicine for last several decades. Ultrasound-mediated delivery of drugs including peptides, genes, steroids and other macromolecules has been recently reported (Liu, J., et al. (1998), Pharm. Res. 15, 918 - 924; Mitragotri, et al. (1996), Pharm. Res. 13, 411 - 420). The earliest report on ultrasound-mediated DNA delivery was the introduction of a plasmid carrying the thymidine kinase gene into cultured fibroblast (Wyber, J.A., et al. (1997), Pharm. Res. 14, 750 - 756). Subsequent studies demonstrated DNA delivery into yeast, plant cells, and mammalian cells (Tata, D.B., et al. (1997), Biochem. Biophys. Res. Commun. 234, 64 – 67; Fechheimer, M., et al. (1987), Proc. Nati. Acad. Sci. USA 84, 8463 - 8567).

Herein the application of ultrasound for gene delivery into animal tissue *in vivo* is disclosed. A 6 to 270-fold increase in systemic gene transfer to primary tumor by sonoporation without any effect on gene expression in non-tumor tissue demonstrates the usefulness of ultrasound for targeted gene delivery to tumors. An approximate 300-fold increase in tumor/normal tissue expression ratio by sonoporation at a 15 microgram plasmid dose shows that the non-specific components of the cationic lipid delivery system can be significantly reduced by a combination of low plasmid dose and sonoporation. This would be useful in increasing the therapeutic index of the cationic lipid based systems for anticancer gene therapies. Sonoporation can be effectively used in combination with tumor targeted ligands or expression systems administered systemically to achieve high levels of tissue-specific gene transfer.

The magnitude of ultrasound enhancement of tumor gene transfer was affected by the sonoporation exposure time, which indicates that selection of appropriate duration of ultrasound exposure is important to achieve gene transfer at desirable level. In contrast, the observation that enhancement of gene transfer depends only weakly on sonoporation energy is useful information, since it permits greater flexibility in designing ultrasound protocols. The effect of ultrasound was inversely related to the time interval between DNA administration and sonoporation application suggesting sonoporation effect is acute and may not involve new protein synthesis.

Co - localization of DNA plasmid with CD31 endothelial cell marker indicates tumor endothelial cells as the primary sites of DNA uptake in sonoporated tumors. A higher DNA uptake in sonoporated tumors compared to non-sonoporated tumors, as determined by PCR and fluorescent microscopy, suggest that the sonoporation enhancement of gene expression in tumors is due to increased DNA uptake. Increase in the perinuclear distribution of DNA

by sonoporation further substantiates that the increase DNA uptake is involved in the effect of sonoporation on gene expression. It has been postulated that the increased permeability of cells to macromolecules by ultrasound treatment is mediated by cavitation, a process of creation and oscillation of gas bubbles in membrane environment (Mitragotri, S., et al. (1995), Science 269, 850 - 853). Membrane lipid disordering from cavitation process forming a transport channel in the disordered lipid region has been proposed as the mechanism of transdermal delivery by sonoporation (Mitragotri, et al. (1996), Pharm. Res. 13, 411 - 420). Increased permeabilization of cell membrane or nuclear membrane to lipid/DNA complexes could be the underlying mechanism of tumor transfection by ultrasound.

The ultrasound treatment appeared to be well tolerated by the animals during and after the treatment as determined by animal survival, mobility and general well being. In human cadaver skin, ultrasound enhanced transdermal drug delivery without causing damage to skin or underlying tissue or altering the permeability properties of the epidermis (Gallo, S.A., et al. (1997), Biophys. J. 72, 2805-2811). The sonoporation treatment does not affect DNA integrity since the size and sequence of plasmid DNA isolated from yeast cells transformed with sonication appeared identical to that obtained from cells transformed in the absence of sonoporation (Liu, J., et al. (1998), Pharm. Res. 15, 918-924).

In summary, ultrasound is a promising new and safe method for tumor targeting of systemically administered genes. A specific enhancement in gene transfer to tumor by sonoporation thus minimizing delivery to normal tissue is critical for the development of safe and effective systemic gene therapy methods for treatment of cancer.

I. Preparation of Formulations

Formulations of nucleic acid molecules can be prepared as disclosed in Example 1. Substitute polymers are selected as determined by application. Generally, a weight/volume ratio is used as exemplified in both of the provided examples.

Delivery and expression of nucleic acids in many formulations, such as in saline, is limited due to degradation of the nucleic acids by cellular components of organisms, such as for instance nucleases. Thus, protection of the nucleic acids when delivered *in vivo* can greatly enhance the resulting expression, and thereby enhance a desired pharmacological or therapeutic effect. It was found that certain types of compounds that interact with a nucleic acid (e.g., DNA) in solution but do not condense the nucleic acid provide *in vivo* protection to the nucleic acid, and correspondingly enhance the expression of an encoded gene product.

Some of these compounds have been discussed in U.S. Patent No. 08/484,777, filed June 7, 1998, International Patent Application No. WO9640958 filed April 23, 1996, U.S. Patent Application Serial Number 60/045,295, filed May 2, 1997, and International Patent Application No. PCT/US95/17038, all of which are incorporated herein by reference in their entirety including any drawings.

The use of delivery systems designed to interact with plasmids and protect plasmids from rapid extracellular nuclease degradation are described in, Mumper, R.J., et al., 1996, *Pharm. Res.* 13:701-709; Mumper, R.J., et al., 1997. Submitted to *Gene Therapy.* A characteristic of the PINC systems is that they are non-condensing systems that allow the plasmid to maintain flexibility and diffuse freely throughout the muscle while being protected from nuclease degradation. While the PINC systems are primarily discussed below, it will be understood that cationic lipid based systems and systems utilizing both PINCS and cationic lipids are also within the scope of the present invention.

A common structural component of the PINC systems is that they are amphiphilic molecules, having both a hydrophilic and a hydrophobic portion. The hydrophilic portion of the PINC is meant to interact with plasmids by hydrogen bonding (via hydrogen bond acceptor or donor groups), Van der Waals interactions, or/and by ionic interactions. For example, PVP and N-methyl-2-pyrrolidone (NM2P) are hydrogen bond acceptors while PVA and Propylene Glycol (PG) are hydrogen bond donors.

All four molecules have been reported to form complexes with various (poly)anionic molecules (Buhler V., BASF Aktiengescellschaft Feinchemie, Ludwigshafen, pp 39-42; Galaev Y, et al., *J. Chrom. A.* 684:45-54 (1994); Tarantino R, et al. *J. Pharm. Sci.* 83:1213-1216 (1994); Zia, H., et al., *Pharm. Res.* 8:502-504 (1991)). The hydrophobic portion of the PINC systems is designed to result in a coating on the plasmid rendering its surface more hydrophobic. Kabanov et al. have described previously the use of cationic polyvinyl derivatives for plasmid condensation designed to increase plasmid hydrophobicity, protect plasmid from nuclease degradation, and increase its affinity for biological membranes (Kabanov, A.V., and Kabanov, V.A., 1995, *Bioconj. Chem.* 6:7-20; Kabanov, A.V., et al., 1991, *Biopolymers* 31:1437-1443; Yaroslavov, A.A., et al., 1996, *FEBS Letters* 384:177-180).

A substantial protective effect is observed; up to at least a one log enhancement of gene expression in rat muscle over plasmid formulated in saline has been demonstrated with these exemplary PINC systems. We have also found that the expression of reporter genes in muscle using plasmids complexed with the PINC systems was more reproducible than when

the plasmid was formulated in saline. For example, the coefficient of variation for reporter gene expression in muscle using plasmid formulated in saline was 96 ± 35% (n = 20 studies; 8-12 muscles/study) whereas with coefficient of variation with plasmids complexed with PINC systems was 40 ± 19% (n = 30 studies; 8-12 muscles/study). The high coefficient of variation for reporter gene expression with plasmid formulated in saline has been described previously (Davis, H.L., et al., 1993, *Hum. Gene Ther.* 4:151-9). In addition, in contrast with the results for DNA:saline, there was no significant difference in gene expression in muscle when plasmid with different topologies were complexed with polyvinyl pyrrolidone (PVP). This suggests that PVP is able to protect all forms of the plasmid from rapid nuclease degradation.

1. Summary of interactions between a PINC polymer (PVP) and plasmid

We have demonstrated using molecular modeling that an exemplary PINC polymer, PVP, forms hydrogen bonds with the base pairs of a plasmid within its major groove and results in a hydrophobic surface on the plasmid due to the vinyl backbone of PVP. These interactions are supported by the modulation of plasmid zeta potential by PVP as well as by the inhibition of ethidium bromide intercalation into complexed plasmid. We have correlated apparent binding between PVP and plasmid to pH and salt concentration and have demonstrated the effect of these parameters on beta-gal expression after intramuscular injection of plasmid/PVP complexes (Mumper, R.J., et al., 1997. Submitted to *Gene Therapy*). A summary of the physico-chemical properties of plasmid/PVP complexes is listed in Table I below.

Table I: Summary of the Physico-Chemical Properties of Plasmid/PVP Complexes

Method	Result
Molecular modeling	Hydrogen bonding and hydrophobic plasmid surface observed
Fourier-transformed Infra-red	Hydrogen bonding demonstrated
DNase I challenge	Decreased rate of plasmid degradation in the presence of PVP
Microtitration Calorimetry	Positive heats of reaction indicative of an endothermic process
Potentiometric titration	One unit pH drop when plasmid and PVP are complexed
Dynamic Dialysis	Rate of diffusion of PVP reduced in the presence of plasmid
Zeta potential modulation	Surface charge of plasmid decreased by PVP
Ethidium bromide Intercalation	Ethidium bromide intercalation reduced by plasmid/PVP complexation
Osmotic pressure	Hyper-osmotic formulation (i.e., 340 mOsm/kg H_2O)
Luminescence Spectroscopy	Plasmid/PVP binding decreased in salt and/or at pH 7

2. Histology of expression in muscle

Immunohistochemistry for beta-gal using a slide scanning technology has revealed the uniform distribution of beta -gal expression sites across the whole cross-sections of rat tibialis muscles. Very localized areas were stained positive for beta -gal when CMV- beta-gal plasmid was formulated in saline. Beta -gal positive cells were observed exclusively around the needle tract when plasmid was injected in saline. This is in agreement with previously published results (Wolff, J.A., et al., 1990, *Science* 247:1465-68; Davis, H.L., et al., 1993, *Hum. Gene Ther.* 4:151-9; Davis, H.L., et al., 1993, *Hum. Gene Ther.* 4:733-40).

In comparison, immunoreactivity for beta-gal was observed in a wide area of muscle tissue after intramuscular injection of CMV- beta-gal plasmid/PVP complex (1:17 w/w) in 150 mM NaCl. It appeared that the majority of positive muscle fibers were located at the edge of muscle bundles. Thus, staining for beta-gal in rat muscle demonstrated that, using a plasmid/PVP complex, the number of muscle fibers stained positive for beta-gal was approximately 8-fold greater than found using a saline formulation. Positively stained muscle fibers were also observed over a much larger area in the muscle tissue using the plasmid/PVP complex providing evidence that the injected plasmid was widely dispersed after intramuscular injection.

One conclusion is that the enhanced plasmid distribution and expression in rat skeletal muscle was a result of both protection from extracellular nuclease degradation due to complexation and hyper-osmotic effects of the plasmid/PVP complex. However, Dowty and Wolff et al. have demonstrated that osmolarity, up to twice physiologic osmolarity, did not significantly affect gene expression in muscle (Dowty, M.E., and Wolff, J.A. In: J.A. Wolff

(Ed.), 1994, *Gene Therapeutics: Methods and Applications of Direct Gene Transfer.* Birkhauser, Boston, pp. 82-98). This suggests that the enhanced expression of plasmid due to PVP complexation is most likely due to nuclease protection and less to osmotic effects. Further, the surface modification of plasmids by PVP (e.g., increased hydrophobicity and decreased negative surface charge) may also facilitate the uptake of plasmids by muscle cells.

3. Structure-activity relationship of PINC polymers

A linear relationship between the structure of a series of co-polymers of vinyl pyrrolidone and vinyl acetate and the levels of gene expression in rat muscle has been found. Also, the substitution of some vinyl pyrrolidone monomers with vinyl acetate monomers in PVP results in a co-polymer with reduced ability to form hydrogen bonds with plasmids. The reduced interaction subsequently led to decreased levels of gene expression in rat muscle after intramuscular injection. The expression of beta-gal decreased linearly ($R = 0.97$) as the extent of vinyl pyrrolidone monomer (VPM) content in the co-polymers decreased.

These data demonstrate that pH and viscosity are not the most important parameters effecting delivery of plasmid to muscle cells since these values were equivalent for all complexes. These data suggest that enhanced binding of the PINC polymers to plasmid results in increased protection and bioavailability of plasmid in muscle.

4. Additional PINC systems

The structure-activity relationship described above can be used to design novel co-polymers that will also have enhanced interaction with plasmids. It is expected that there is "an interactive window of opportunity" whereby enhanced binding affinity of the PINC systems will result in a further enhancement of gene expression after their intramuscular injection due to more extensive protection of plasmids from nuclease degradation. It is expected that there will be an optimal interaction beyond which either condensation of plasmids will occur or "triplex" type formation, either of which can result in decreased bioavailability in muscle and consequently reduced gene expression.

As indicated above, the PINC compounds are generally amphiphilic compounds having both a hydrophobic portion and a hydrophilic portion. In many cases the hydrophilic portion is provided by a polar group. It is recognized in the art that such polar groups can be provided by groups such as, but not limited to, pyrrolidone, alcohol, acetate, amine or heterocyclic groups such as those shown on pp. 2-73 and 2-74 of CRC Handbook of

Chemistry and Physics (72nd Edition), David R. Lide, editor, including pyrroles, pyrazoles, imidazoles, triazoles, dithiols, oxazoles, (iso)thiazoles, oxadiazoles, oxatriazoles, diaoxazoles, oxathioles, pyrones, dioxins, pyridines, pyridazines, pyrimidines, pyrazines, piperazines, (iso)oxazines, indoles, indazoles, carpazoles, and purines and derivatives of these groups, hereby incorporated by reference.

Compounds also contain hydrophobic groups which, in the case of a polymer, are typically contained in the backbone of the molecule, but which may also be part of a non-polymeric molecule. Examples of such hydrophobic backbone groups include, but are not limited to, vinyls, ethyls, acrylates, acrylamides, esters, celluloses, amides, hydrides, ethers, carbonates, phosphazenes, sulfones, propylenes, and derivatives of these groups. The polarity characteristics of various groups are quite well known to those skilled in the art as illustrated, for example, by discussions of polarity in any introductory organic chemistry textbook.

The ability of such molecules to interact with nucleic acids is also understood by those skilled in the art, and can be predicted by the use of computer programs which model such intermolecular interactions. Alternatively or in addition to such modeling, effective compounds can readily be identified using one or more of such tests as: 1) determination of inhibition of the rate of nuclease digestion, 2) alteration of the zeta potential of the DNA, which indicates coating of DNA, or 3) inhibition of the ability of intercalating agents, such as ethidium bromide to intercalate with DNA.

5. Targeting Ligands

In addition to the nucleic acid/PINC complexes described above for delivery and expression of nucleic acid sequences, in particular embodiments it is also useful to provide a targeting ligand in order to preferentially obtain expression in particular tissues, cells, or cellular regions or compartments.

Such a targeted PINC complex includes a PINC system (monomeric or polymeric PINC compound) complexed to plasmid (or other nucleic acid molecule). The PINC system is covalently or non-covalently attached to (bound to) a targeting ligand (TL) which binds to receptors having an affinity for the ligand. Such receptors may be on the surface or within compartments of a cell. Such targeting provides enhanced uptake or intracellular trafficking of the nucleic acid.

The targeting ligand may include, but is not limited to, galactosyl residues, fucosal residues, mannosyl residues, carnitine derivatives, monoclonal antibodies, polyclonal antibodies, peptide ligands, and DNA-binding proteins. Examples of cells which may

usefully be targeted include, but are not limited to, antigen-presenting cells, hepatocytes, myocytes, epithelial cells, endothelial cells, and cancer cells.

Formation of such a targeted complex is illustrated by the following example of covalently attached targeting ligand (TL) to PINC system:

TL-PINC + Plasmid ----------> TL-PINC::::::Plasmid

Formation of such a targeted complex is also illustrated by the following example of non-covalently attached targeting ligand (TL) to PINC system

TL::::::PINC + Plasmid --------> TL::::::PINC::::::Plasmid

or alternatively,

PINC + Plasmid ----------> PINC::::::::Plasmid + TL ----------> TL::::::PINC::::::::Plasmid

In these examples :::::::: is non-covalent interaction such as ionic, hydrogen-bonding, Van der Waals interaction, hydrophobic interaction, or combinations of such interactions.

A targeting method for cytotoxic agents is described in Subramanian et al., International Application No. PCT/US96/08852, International Publication No. WO 96/39124, hereby incorporated by reference. This application describes the use of polymer affinity systems for targeting cytotoxic materials using a two-step targeting method involving zip polymers, i.e., pairs of interacting polymers. An antibody attached to one of the interacting polymers binds to a cellular target. That polymer then acts as a target for a second polymer attached to a cytotoxic agent. As referenced in Subramanian et al., other two-step (or multi-step) systems for delivery of toxic agents are also described.

In another aspect, nucleic acid coding sequences can be delivered and expressed using a two-step targeting approach involving a non-natural target for a PINC system or PINC-targeting ligand complex. Thus, for example, a PINC-plasmid complex can target a binding pair member which is itself attached to a ligand which binds to a cellular target (e.g., a MAB). Binding pairs for certain of the compounds identified herein as PINC compounds as identified in Subramanian et al. Alternatively, the PINC can be complexed to a targeting

ligand, such as an antibody. That antibody can be targeted to a non-natural target which binds to, for example, a second antibody.

II. Administration

Administration as used herein refers to the route of introduction of a plasmid or carrier of DNA into the body. Administration can be directly to a target tissue or by targeted delivery to the target tissue after systemic administration. In particular, the present invention can be used for treating conditions by administration of the formulation to the body in order to establish controlled expression of any specific nucleic acid sequence within tissues at certain levels that are useful for gene therapy.

The preferred means for administration of vector (plasmid) and use of formulations for delivery are described above and involve sonoporation of the target cells.

The route of administration of any selected vector construct will depend on the particular use for the expression vectors. In general, a specific formulation for each vector construct used will focus on vector delivery with regard to the particular targeted tissue, the sonoporation delivery parameters, followed by demonstration of efficacy. Delivery studies will include uptake assays to evaluate cellular uptake of the vectors and expression of the DNA of choice. Such assays will also determine the localization of the target DNA after uptake, and establishing the requirements for maintenance of steady-state concentrations of expressed protein. Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Muscle cells have the unique ability to take up DNA from the extracellular space after simple injection of DNA particles as a solution, suspension, or colloid into the muscle. Expression of DNA by this method can be sustained for several months.

Delivery of formulated DNA vectors involves incorporating DNA into macromolecular complexes that undergo endocytosis by the target cell. Such complexes may include lipids, proteins, carbohydrates, synthetic organic compounds, or inorganic compounds. Preferably, the complex includes DNA, a cationic lipid, and a neutral lipid in particular proportions. The characteristics of the complex formed with the vector (size, charge, surface characteristics, composition) determines the bioavailability of the vector within the body. Other elements of the formulation function as ligand which interact with specific receptors on the surface or interior of the cell. Other elements of the formulation function to enhance entry into the cell, release from the endosome, and entry into the nucleus.

Delivery can also be through use of DNA transporters. DNA transporters refers to molecules which bind to DNA vectors and are capable of being taken up by epidermal cells. DNA transporters contain a molecular complex capable of noncovalently binding to DNA and efficiently transporting the DNA through the cell membrane. It is preferable that the transporter also transport the DNA through the nuclear membrane. See, *e.g.*, the following applications all of which (including drawings) are hereby incorporated by reference herein: (1) Woo et al., U.S. Serial No. 07/855,389, entitled "A DNA Transporter System and Method of Use", filed March 20, 1992, now abandoned; (2) Woo et al., PCT/US93/02725, International Publ. WO93/18759, entitled "A DNA Transporter System and Method of Use", (designating the U.S. and other countries) filed March 19, 1993; (3) continuation-in-part application by Woo et al., entitled "Nucleic Acid Transporter Systems and Methods of Use", filed December 14, 1993, U.S. Patent No. 6,033,884; (4) Szoka et al. , U.S. Serial No. 07/913,669, entitled "Self-Assembling Polynucleotide Delivery System", filed July 14, 1992 and (5) Szoka et al., PCT/US93/03406, International Publ. WO93/19768 entitled "Self-Assembling Polynucleotide Delivery System", (designating the U.S. and other countries) filed April 5, 1993.

A DNA transporter system can consist of particles containing several elements that are independently and non-covalently bound to DNA. Each element consists of a ligand which recognizes specific receptors or other functional groups such as a protein complexed with a cationic group that binds to DNA. Examples of cations that may be used are spermine, spermine derivatives, histone, cationic peptides and/or polylysine.

One element is capable of binding both to the DNA vector and to a cell surface receptor on the target cell. Examples of such elements are organic compounds that interact with the asialoglycoprotein receptor, the folate receptor, the mannose-6-phosphate receptor, or the carnitine receptor.

A second element is capable of binding both to the DNA vector and to a receptor on the nuclear membrane. The nuclear ligand is capable of recognizing and transporting a transporter system through a nuclear membrane. An example of such ligand is the nuclear targeting sequence from SV40 large T antigen or histone.

A third element is capable of binding to both the DNA vector and to elements that induce episomal lysis. Examples include inactivated virus particles such as adenovirus, peptides related to influenza virus hemagglutinin, or the GALA peptide described in the Szoka patent cited above.

Administration may also involve lipids as described in preferred embodiments above. The lipids may form liposomes which are hollow spherical vesicles composed of lipids arranged in unilamellar, bilamellar, or multilamellar fashion and an internal aqueous space for entrapping water soluble compounds, such as DNA, ranging in size from 0.05 to several microns in diameter. Lipids may be useful without forming liposomes. Specific examples include the use of cationic lipids and complexes containing DOPE that interact with DNA and with the membrane of the target cell to facilitate entry of DNA into the cell.

The chosen method of delivery should result in expression of the gene product encoded within the nucleic acid cassette at levels that exert an appropriate biological effect. The rate of expression will depend upon the disease, the pharmacokinetics of the vector and gene product, and the route of administration, but should be in the range 0.001-100 mg/kg of body weight/day, and preferably 0.01-10 mg/kg of body weight/day. This level is readily determinable by standard methods. It could be more or less depending on the optimal dosing. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon the disease, delivery vehicle, and efficacy data from clinical trials.

III. DNA Injection Variables

The level of gene delivery and expression or the intensity of an immune response achieved with the present invention can be optimized by altering the following variables discussed herein. An immune response can be measured by, but is not limited to, the amount of antibodies produced for a protein encoded and expressed by the injected nucleic acid molecule.

IV. Nucleic acid based therapy

The present invention can be used to deliver nucleic acid vaccines in a more efficient manner than is conventionally done at the present time. Nucleic acid vaccines, or the use of plasmid encoding antigens or therapeutic molecules such as Human Growth Hormone, has become an area of intensive research and development in the last half decade. Comprehensive reviews on nucleic acid based vaccines have been published (M.A. Liu, et al.(Eds.), 1995, *DNA Vaccines: A new era in vaccinology*, Vol. 772, Ann. NY. Acad. Sci., New York; Kumar, V., and Sercarz, E., 1996, *Nat. Med.* 2:857-859; Ulmer, J.B., et al., (Eds.) *Current Opinion in Immunology*; 8:531-536. Vol. 772, Ann. NY. Acad. Sci., New York). Protective

immunity in an animal model using plasmid encoding a viral protein was first observed in 1993 by Ulmer et al. (Ulmer, J.B., et al., 1993, *Science* 259:1745-1749). Since then, several studies have demonstrated protective immunity for several disease targets and human clinical trials have been started.

Many disease targets have been investigated. Examples include antigens of *Borrelia burgdorferi*, the tick-borne infectious agent for Lyme disease (Luke et al., *J. Infect. Dis.* 175:91-97, 1997), human immunodeficiency virus-1, (Letvin et al., *Proc. Nat. Acad. Sci. USA* 94:9378-9383, 1997), B cell lymphoma (Syrengelas et al., *Nature Medicine*. 2:1038-41, 1996), Herpes simplex virus (Bourne et al., *J. Infectious dis.* 173:800-807, 1996), hepatitis C virus (Tedeschi et al., *Hepatology* 25:459-462, 1997), rabies virus (Xiang et al., *virology*, 209:569-579, 1995), *Mycobacterium tuberculosis* (Lowrie in Genetic Vaccines and Immunotherapeutic Strategies CA Thibeault, ed. Intl Bus Comm, Inc., southborough, MA 01772 pp. 87-122, 1996), and *Plasmodium falciparum* (Hoffman et al., *Vaccine* 15:842-845, 1997). Additionally, nucleic acid based treatment for reducing tumor-cell immunogenicity, growth, and proliferation is indicative of gene therapy for diseases such as tumorigenic brain cancer (Fakhrai et al., Proc. Natl. Acad. Sci., 93:2909-2914, 1996).

An important goal of gene therapy is to affect the uptake of nucleic acid by cells, thereby causing an immune response to the protein encoded by the injected nucleic acid. Uptake of nucleic acid by cells is dependent on a number of factors, one of which is the length of time during which a nucleic acid is in proximity to a cellular surface. The present invention provides formulations that increase the length of time during which a nucleic acid is in proximity to a cellular surface, and penetrate the cell thereby delivering nucleic acid molecules into the cell.

Nucleic acid based vaccines are an attractive alternative vaccination strategy to subunit vaccines, purified viral protein vaccines, or viral vector vaccines. Each of the traditional approaches has limitations that are overcome if the antigen(s) is expressed directly in cells of the body. Furthermore, these traditional vaccines are only protective in a strain-specific fashion. Thus, it is very difficult, and even impossible using traditional vaccine approaches to obtain long lasting immunity to viruses that have several sera types or viruses that are prone to mutation.

Nucleic acid based vaccines offer the potential to produce long lasting immunity against viral epitopes that are highly conserved, such as with the nucleoprotein of viruses. Injecting plasmids encoding specific proteins by the present invention results in increased

immune responses, as measured by antibody production. Thus, the present invention includes new methods of providing nucleic acid vaccines by delivering a formulated nucleic acid molecule with a sonoporation device as described herein.

The efficacy of nucleic acid vaccines is enhanced by one of at least three methods: (1) the use of delivery systems to increase the stability and distribution of plasmid within the muscle, (2) by the expression (or delivery) of molecules to stimulate antigen presentation/transfer, or (3) by the use of adjuvants that may modulate the immune response.

V. Polymeric and non-polymeric formulations for plasmid delivery

The present invention provides polymeric and non-polymeric formulations that address problems associated with injection of nucleic acids suspended in saline. Unformulated (naked nucleic acid molecules) plasmids suspended in saline have poor bioavailability in muscle due to rapid degradation of plasmid by extracellular nucleases. One possible approach to overcome the poor bioavailability is to protect plasmid from rapid nuclease degradation by for example condensing the plasmid with commonly used cationic complexing agents. However, due to the physiology of the muscle, the use of rigid condensed particles containing plasmid for efficient transfection of a larger number of muscle cells has not been successful to date. Cationic lipid and polylysine plasmid complexes do not cross the external lamina to gain access to the caveolae and T tubules ([Wolff, J.A., et al., 1992, *J. Cell. Sci.* 103:1249-1259).

Thus, the strategy identified for increasing the bioavailability of plasmid in muscle was to: protect plasmid from rapid extracellular nuclease degradation, disperse and retain intact plasmid in the muscle and/or tumor, and facilitate the uptake of plasmid by muscle and/or tumor cells. A specific method of accomplishing this, which preferably is used in conjunction with sonoporation is the use of protective, interactive, non-condensing systems (PINC).

VI. Diseases and Conditions for Intramuscular Plasmid Delivery

The present invention described herein can be utilized for the delivery and expression of many different coding sequences. In particular, the demonstrated effectiveness for the PINC systems (PCT Application No. PCT/US95/17038, WO9621470) for delivery to muscle indicate that such formulations are effective for delivery and sonoporation of a large variety

of coding sequences to muscle and/or tumor. As transforming muscle and other cells has been shown to be effective, in an additional aspect of the invention tumor cells are also targeted for sonoporation. Hence, the present invention provides methods for treating cancerous conditions associated with the formation of tumors or aggregated cell colonies such as those found in conditions such as skin cancer and the like. Specific suggestions for delivery and sonoporation of coding sequences to muscle cells include those summarized in Table 2 below.

Table 2: Applications for Plasmid-Based Gene Therapy by Intramuscular Injection

Muscle and nerve disorders	**References are numbered as they are cited in U.S. Application No. PCT/US96/05679, which has been incorporated by reference in its entirety.**
Duchenne's muscular dystrophy	Acsadi 1991 [5], Karpati 1993 [6], Miller 1995 [7]
Myotrophic disorders (IGF-I)	Coleman 1997 [8], Alila 1997 [9]
Neurotrophic disorders (IGF-I)	Alila 1997 [9], Rabinovsky 1997 [10]
Secretion of expressed protein into the systemic circulation	
Hemophilias A and B	Anwer 1996 [11], Kuwahara-Rundell 1994 [12], Miller 1994 [13]
Erythropoietin-responsive	Tripathy 1996 [14]
Pituitary dwarfism	Anwer 1996 [11], Dahler 1994 [15]
1-Antitrypsin deficiency	Levy 1996 [16]
Autoimmune and Inflammatory diseases	Raz 1993 [17]
Hypercholesterolema	Fazio 1994 [18]
Hypotension	Ma 1995 [19]
Hypertension	Xiong 1995 [20]
Nucleic acid vaccines	
Herpes Simplex Virus	Manickan 1995 [21], Ghiasi 1995 [22], McClements 1996 [23], Kriesel 1996 [24]
Hepatitis B Virus	Davis 1993 [25], Davis 1994 [26], Davis 1996 [27]
Influenza Virus	Donnelly 1995 [28], Ulmer 1993 [29], Ulmer 1994 [30]
Tuberculosis	Lowrie 1994 [31], Tascon, 1996 [32]
Human Immunodeficiency Virus	Shiver 1995 [33], Coney 1994 [34], Wang 1993 [35]
Cancer	Raz 1993 [17], Russell 1994 [36]
Maleria	Hoffman 1995 [37], Sedegah 1994 [38]
Hepatitis C virus	Major 1995 [39], Lagging 1995 [40]
Flavivirus	Phillpotts 1996 [41]
Cytomegalovirus	Pande 1995 [42]
Salmonella typhi	Lopez-Macias 1995 [43]
Mycoplasma pulmonis	Lai 1995 [44]
Rabies virus	Xiang 1995 [45]

Diseases to Be Treated.

The condition or disease preferably is a cancer, such as epithelial glandular cancer, including adenoma and adenocarcinoma; squamous and transitional cancer, including polyp, papilloma, squamous cell and transitional cell carcinoma; connective tissue cancer, including tissue type positive, sarcoma and other (oma's); hematopoietic and lymphoreticular cancer, including lymphoma, leukemia and Hodgkin's disease; neural tissue cancer, including

neuroma, sarcoma, neurofibroma and blastoma; mixed tissues of origin cancer, including teratoma and teratocarcinoma. Other cancerous conditions that are applicable to treatment include cancer of any of the following: adrenal gland, anus, bile duct, bladder, brain tumors: adult, breast, cancer of an unknown primary site, carcinoids of the gastrointestinal tract, cervix, childhood cancers, colon and rectum, esophagus, gall bladder, head and neck, islet cell and other pancreatic carcinomas, Kaposi's sarcoma, kidney, leukemia, liver, lung: non-small cell, lung: small cell, lymphoma: AIDS-associated, lymphoma: Hodgkin's disease, Lymphomas: non-Hodgkin's disease, melanoma, mesothelioma, metastatic cancer, multiple myeloma, ovary, ovarian germ cell tumors, pancreas, parathyroid, penis, pituitary, prostate, sarcomas of bone and soft tissue, skin, small intestine, stomach, testis, thymus, thyroid, trophoblastic disease, uterus: endometrial carcinoma, uterus: uterine sarcomas, vagina, or vulva. The composition preferably is administered and sonoporation and may require, as needed, exposure of the tissue to be treated by surgical means as determined by a certified professional.

Examples

The following examples are offered by way of illustration and are not intended to limit the scope of the invention in any manner. One of ordinary skill in the art would recognize that the various molecules and/ or amounts disclosed in the examples could be adjusted or substituted by larger amounts (for larger scaled experiments) or by inclusion of a different transfection facilitating agent. It would also be recognized that the delivery targets and/ or amounts delivered in the examples could be adjusted or substituted by selecting different muscles for injection, injection into tumors or nodes, or modifying the other sonoporation factors described herein.

Example 1

Experimental Protocols

Plasmid Construction and Preparation

The expression plasmid pCMV-CAT contained a CMV enhancer and promoter, an SV40 intron, a chloramphenicol acetyltransferase (CAT) gene and a SV40 late poly A signal. The expression plasmid pCMV-IL-12 contained two transcription units in tandem, one for the p35 subunit and one for the p40 subunit, each transcription unit included a CMV enhancer

and promoter, a synthetic intron, an IL-12 subunit coding sequence and a human growth hormone poly A signal. The pDNA was isolated and purified from *Escherichia coli* by chromatography. The purity of pDNA preparations was determined by 1% agarose gel electrophoresis followed by SYBR™ Green (Molecular Probes, Eugene, OR) staining, and DNA concentration was measured by UV absorption at 260 nm. The percentage of supercoiled pDNA and $OD_{260/280}$ ratios of these pDNA preparations was in the range of 70-95% and 1.8-1.9, respectively. Endotoxin levels of pDNA preparations were determined using the chromogenic limulus amebocyte lysate assay (Chromogenic End-Point, LAL BioWhittaker, Walkersville, MD) and were <50 endotoxin units (EU)/mg.

Preparation of Plasmid/Cationic Lipid Complexes:

Liposomes containing N-[1-(2-3-dioleyloxy)propyl)]-N-N-N-trimethylammonium chloride (DOTMA) and cholesterol (CHOL) (Avanti Polar Lipids, Inc., Alabaster, AL) were prepared at 4:1 DOTMA:CHOL molar ratio. Briefly, lipids were mixed in chloroform and evaporated to a thin film in a 50 ml round bottom flask using a rotary evaporator. The film was hydrated in sterile water, probe-sonicated, centrifuged at 32000 x g for 30 minutes, and sterile filtered to obtain small unilamelar vesicles (SUV). DOTMA:CHOL/plasmid complexes were formed in 10% lactose by controlled mixing of liposomes with DNA plasmid at 3:1 (+/-) charge ratio unless stated otherwise. The plasmid concentration in the formulation was 300 microgram/ml. For 10 microgram, 15 microgram, and 45 microgram doses, serial dilutions with 10% lactose were made to obtain DNA concentrations of 33 microgram/ml, 50 microgram /ml, and 150 microgram /ml, respectively.

In Vivo Gene Transfer and Ultrasound Application

Subcutaneous solid tumors were created in 6 - 8 week old female C3H mice (20 - 22 g) (Charles River Laboratories Raleigh, NC) by s.c. injection of 4×10^5 squamous carcinoma cells. The average tumor size before DNA injection was typically in the range of ~30 - 40 mm^3 (6-7 days after implantation).

pCMV-CAT was complexed with DOTMA:CHOL (4:1, mol/mol) with cationic lipid to plasmid ratio of 3:1 (+/-) and administered intravenously into the tail vein of mice. Immediately after the injection of transfection complexes, tumors were covered with Spectrogel 50 conducting gel (Spectrogel XX) and sonoporated for 1, 2, 5, or 15 minutes at 1000 Hz input frequency and 0.5 to 1.50 W/cm^2 output intensity using a sonoporation device

(ImaRx, Tucson, AZ). Tumors and lungs were harvested 18 h after DNA administration, immediately frozen in liquid N_2, and stored at minus 80 C.

Assay for Gene Expression in Mouse Tissue

Tumors and lungs were homogenized in 0.35 ml and 1 ml TENT (Tris 10 mM, EDTA 1 mM, NaCl 0.1 M, Triton X-100 0.5 %)buffer, respectively. The tissue homogenate was centrifuged at 10,000 x g for 15 min and supernatant was assayed for the gene product, for example CAT or IL-12, using a specific ELISA (Boehringer Mannheim, Indianapolis, IN).

Distribution of Lipid/Plasmid Complexes in Tumor

Psoralen-fluorescent-labeled pCMV-CAT was complexed with DOTMA:CHOL (4:1, mol/mol) at 3:1 (+/-) lipid/plasmid charge ratio and injected into s.c. tumor bearing mice via tail vein at a dose of 90 μg DNA/mouse. The tumors were sonoporated at 1.5 W/cm^2 for various periods immediately before and after DNA injection. Immediately following the experimental period, animals were anesthetized by intraperitoneal administration of a mixture of ketamine (42.8 mg/ml), xylazine (8.6 mg/ml) and acepromazine (1.4 mg/ml) at a dose of 0.5-0.7 ml/kg, and whole-body perfusion was performed with 1% BSA/PBS solution to clear entrapped blood. Tumors were removed and embedded in O.C.T embedding medium. Tissue cryosections (5 micrometers) were examined for plasmid fluorescence with an Olympus BX-60 fluorescence microscope (Melville, New York) and photographed using Montague spot camera.

CD31 Immunostaining of Tumor Endothelium

5 micrometer tumor paraffin cryosections (JUNG CM3000) were first incubated with a rat anti-mouse CD31 antibody (Pharmingen, San Diego, CA), and then with a rabbit anti-rat IgG labeled with Texas Red fluorophore (Vector Laboratories, Inc., Burlingame, CA). The tumor sections were fixed in formaldehyde and counterstained with Vectashield™ mounting medium containing DAPI to highlight nuclei. Controls included unstained sections and incubation with secondary antibody alone. Tissue sections were viewed using an Olympus BX-60 fluorescence microscope and photographed using a Montague spot camera.

Plasmid Isolation and Quantification

Tissues were digested by incubation with digestion buffer (100 mM NaCl, 10 mM

Tris-HCl, [pH 8.0], 25 mM EDTA [pH 8.0], 0.5% SDS, and proteinase K [0.1 milligram/ml]) at 50° C. The samples were extracted with an equal volume of Tris-buffered phenol (pH 8.0), followed by extraction with chloroform:isoamyl alcohol (24:1, v/v) and ethanol precipitation. The DNA precipitates were dissolved in TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA), and DNA concentration was measured by UV absorption at 260 nm. The amount of plasmid DNA associated with the tissue was quantified by a polymerase chain reaction (PCR) assay using Taqman PCR (Perkin-Elmer, Foster City, CA). For detection of pCMV-CAT plasmid DNA, the primers used in the reaction were a forward primer, 5'-TGA CCT CCA TAG AAG ACA CCG GGA C-3' (Genosys Biotechnologies, The Woodlands, TX), which primes in the CMV 5' untranslated region (UTR), and a reverse primer, 5' AGG CCG TAA TAT CCA GCT GAACG-3', which primes in the CAT coding region. The probe sequence was 5'-CCA GCC TCC GGA CTC TAG AGG A-3'. The initial copy numbers of unknown samples were determined by using an Applied Biosystem 7700 sequence detector to compare them with a standard curve generated from purified pCMV-CAT of known initial copy numbers.

Effect of Sonoporation on Gene Transfer in s.c. Tumors After Systemic Administration

Systemic administration of 15 μg pCMV-CAT complexed with DOTMA:CHOL (4:1 mol/mol) at 3:1 (+/-) lipid/plasmid charge ratio into tail vein of SCCVII s.c. tumor bearing mice yielded low levels (4.6 +/-10.4 pg/g tumor) of CAT. To test the effects of sonoporation, a 15, 45, or 90 microgram plasmid dose of DOTMA:Chol formulated CAT plasmid was administered by tail vein to SCCVII tumor bearing mice. Tumors were sonoporated at 1.5 W/cm^2 for 5 min immediately after plasmid injection. CAT expression levels in tumor (A) and lung (B) were measured 18-20 h after DNA administration. Ultrasound treatment (1.5 W/cm^2 for 5 min) of tumors following systemic administration of transfection complex increased the levels of CAT expression up to 270-fold over control (Fig. 1). The data is expressed as mean ± S.D., n=5. The enhancement of gene expression by sonoporation was observed at all DNA doses tested (Fig. 1). The magnitude of sonoporation enhancement of gene expression over control at 15, 45, and 90 micrograms DNA was 270, 40, and 6-fold, respectively.

The sonoporation enhancement of gene expression was tumor specific since no increase was observed in lung (Fig. 1B). The sonoporation treatment increased the tumor/non-tumor expression ratio (Fig. 2).

The tumor/lung expression ratios in non-sonoporated animals were 0.001, 0.0001, and

0.0009 at 15, 45, and 90 microgram DNA, respectively. In sonoporated animals the tumor/lung ratios increased to 0.3, 0.01, and 0.004 at 15, 45, and 90 microgram dose, respectively.

The magnitude of sonoporation enhancement of tumor gene transfer was dependent on the duration of sonoporation (Fig. 3). For the data presented in Figure 3, PCMV-CAT was complexed with DOTMA:CHOL at 90 μg DNA dose and administered into tumor bearing mice by tail vein injection. Tumors were sonoporated (1.5 W/cm^2) for 1, 2, 5, or 15 minutes after plasmid injection. Tumors were collected 18-20 h later for measurement of CAT expression (mean +/- S.D., n = 4 - 5). The CAT levels increased as the sonoporation period was increased. Maximal increase was observed when sonoporated for 5 minutes after plasmid injection. Modulation of sonoporation output between 1 - 2 W/cm^2 did not significantly alter the levels of CAT expression in tumor or lung. The levels of CAT expression were also sensitive to the time interval between plasmid administration and sonoporation.

For the experiment presented in Figure 4, PCMV -CAT was complexed with DOTMA:CHOL at 90 μg DNA dose and administered into tumor bearing mice by tail vein injection. Tumors were sonoporated for 5 minutes (1.5 W/cm^2) beginning 0.1, 1, 2, 5, 15 or 30 min after plasmid injection. Tumor and lung were collected 18 - 20 h later for measurement of CAT expression (mean +/- S.D., n = 4 - 5). Best results were achieved when sonoporation was performed within 5 min of plasmid injection (Fig. 4).

The effect of ultrasound before plasmid administration was also examined as shown in Figure 5. Application (1.5 W/cm^2 for 2 min) five minutes before DNA (90 microgram) injection increased CAT expression from 0.28±0.09 nanogram/gram to 0.93±0.21 nanogram/gram tumor. Pretreatment of tumors (1.5 W/cm^2 for 2 min) 30 min before DNA administration did not change expression levels (control: 0.29±0.072; sonoporated 0.308±0.094 ng/g tumor.

Measurement of plasmid DNA by qPCR showed 3-fold higher plasmid uptake in sonoporated tumors compared to control tumors (Fig. 5). Figure 5 presents the quantitation of CAT plasmid (mean +/- S.D., n = 5) in mice with s.c. tumors injected with 90 μg of pCMV-CAT complexed with DOTMA:CHOL via tail vein injection, tissue were collected 1 h later.

Sonoporation did not effect plasmid uptake by lung. Psoralen-fluorescein-labeled pCMV-CAT complexed with DOTMA:CHOL (4:1 mol/mol) at 3:1 (+/-) lipid/plasmid charge ratio at 90 μg dose was administered into s.c. tumor bearing mice by tail vein injection.

Tumors were sonoporated at 1.5 W/cm^2 for 5 min immediately after plasmid injection. Tumors were collected 15 min after plasmid injection and tissue cryosections (5 micrometers) were prepared and examined under a fluorescence microscope for distribution of fluorescent plasmid (green). Nuclei are stained in blue with DAPI. The sections were counterstained with a rhodamine labeled anti-CD31 endothelial cell marker. The plasmid fluorescence appeared to be higher in sonoporated tumors compared to non-sonoporated tumors. Sonoporation appeared to increase the perinuclear distribution of the DNA when compared with the control. Co-localization of fluorescent plasmid and antibody to endothelial cell surface marker demonstrates the endothelial localization of sonoporated DNA. The sonoporation treatment did not produce any adverse effect at the sonoporation site or on general well-being of the animals.

Expression of IL-12 gene and inhibition of tumor growth

The combination of ultrasound treatment and systemic delivery of DNA plasmid by liposomes was examined for expression and biological activity of an anti-cancer gene. Figure 6 shows the effect of ultrasound treatment on IL-12 expression in s.c. tumors. Transfection complexes were formed with an IL-12 expression plasmid complexed with DOTMA:CHOL liposomes and systemically administered into tumor bearing mice by tail vein injection Tumors were sonoporated for 5 min at 0.5 W/cm^2 or 1.5 W/cm^2 immediately following administration of a 10 microgram plasmid dose. Tissues were collected 18-20 h later for measurement of IL-12 (mean ±S.D., n=5). The IL-12 levels in ultrasound treated tumors at 0.5 W/cm^2 and 1.5 W/cm^2 were 0.44 ±0.18 ng/g and 0.52±0.31 ng/g tumor, respectively. IL-12 levels in non-sonoporated tumors were 0.064 - 0.066 ng/g. As shown in Fig. 6, systemic tail vein injection of DOTMA:CHOL/pCMV-IL-12 complexes followed by local sonoporation resulted in preferential IL-12 expression in tumors.

A concern arose that increased gene transfer may be the result of physically manipulating the tumor and not due to the application of ultrasound. An experiment was designed to address this concern in which transfection complexes were administered intravenously and the gel and ultrasound device were applied without administration of an ultrasound pulse. Comparisons were made with control and ultrasound treated groups. Only the tumors to which ultrasound was applied showed increased gene expression compared to untreated tumors and tumors that were manipulated with gel and the ultrasound probe (results not shown).

The effect of IL-12 protein levels on primary tumor growth was determined. Figure 7 shows the effect of ultrasound treatment on inhibition of tumor growth following systemic administration of IL-12 transfection complexes. The tumors were sonoporated for 2 min at 1.5 W/cm^2 followed by a single tail vein injection of DOTMA:CHOL transfection complexes containing 10 microgram of IL-12 plasmid or CAT plasmid. Control animals received 10% lactose. Tumor size was measured 14 days after plasmid injection. Bars with different superscripts are statistically different ($p< 0.05$, mean ± S.D., n=5) as determined by one-way ANOVA and Duncan's multiple range test.

As shown in Fig. 7, the combination of systemic administration of IL-12 transfection complexes and ultrasound treatment produced a significant inhibition of tumor growth. The combination of IL-12 transfection complex systemic administration and ultrasound treatment yielded a 65% reduction in tumor growth rate compared to the lactose control group. Tumor inhibition from combination treatment was significantly higher than that obtained with IL-12 gene treatment alone. The effect of ultrasound treatment on tumor growth was specific to IL-12 gene since no effect of ultrasound treatment was observed in lactose or CAT gene injected group.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting

essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example, if X is described as selected from the group consisting of bromine, chlorine, and iodine, claims for X being bromine and claims for X being bromine and chlorine are fully described.

Those references not previously incorporated herein by reference, including both patent and non-patent references, are expressly incorporated herein by reference for all purposes. Other embodiments are within the following claims.

Claims

What is claimed is:

1. Use of a complex, comprising a nucleic acid molecule comprising and capable of functionally expressing a gene complexed to a transfection facilitating agent, for preferential delivery and expression of said nucleic acid into a cell located in a tumor, wherein said preferential delivery and expression is caused by sonoporation of the tumor.

2. The use of claim 1, wherein said gene encodes a gene product selected from the group comprising: immunomodulators, tumor antigens, growth regulatory factors, tumor angiogenesis regulatory factors and combinations thereof.

3. The use of claim 2 wherein the complex is administered systemically.

4. The use of claim 3 wherein the transfection facilitating agent contains a cationic lipid.

5. The use of claim 4, wherein said transfection facilitating agent further comprises cholesterol.

6. The use of claim 5, wherein said cationic lipid comprises DOTMA and said DOTMA and said cholesterol are present in a 4:1 molar ratio of DOTMA to cholesterol.

7. The use of claim 1 wherein the transfection facilitating agent comprises DOTMA/CHOL in a 4:1 molar ratio and wherein the complex comprises nucleic acid to transfection facilitating agent in an overall 1:3 (-/+ charge) ratio.

8. The use of claim 2, wherein said preferential expression of said nucleic acid molecule is increased between about 6 and about 270 fold relative to expression of said nucleic acid molecule after systemic delivery of said complex to the tumor without sonoporation.

9. The use of claim 1, wherein the tumor is sonoporated for between about one and about fifteen minutes.

10. The use of claim 3, wherein the tumor is sonoporated within about ten minutes of said systemic administration of said complex.

11. The use of claim 10, wherein the tumor is sonoporated within about ten minutes after said systemic administration of said complex.

12. The use of claim 2, wherein said immunomodulator is IL-12.

13. A commercial package comprising a nucleic acid molecule comprising and capable of functionally expressing a gene complexed to a transfection facilitating agent, and a written matter associated therewith, wherein the written matter states that that complex is to be used for preferential delivery and expression of said nucleic acid into a cell located in a tumor by sonoporation of the tumor within about ten minutes of systemic administration of the complex.

14. The commercial package of claim 13 wherein the gene encodes a immunomodulator and said transfection facilitating agent comprises a cationic lipid.

15. The commercial package of claims14 wherein the immunomodulator is IL-12.

16. The commercial package of claim 13 wherein the transfection facilitating agent comprises DOTMA/CHOL in a 4:1 molar ratio and wherein the nucleic acid to transfection facilitating agent is complexed in an overall 1:3 (-/+ charge) ratio.

17. A method for delivering a nucleic acid molecule to a tumor in vivo, comprising the steps of:

administering in vivo a complex comprising said nucleic acid molecule and a transfection facilitating agent; and

sonoporating the tumor.

18. The method of claim 17, wherein said nucleic acid molecule is expressed and encodes a gene product selected from the group comprising: immunomodulators, tumor antigens, growth regulatory factors, angiogenesis regulatory factors and combinations thereof.

19. The method of claim 17 wherein said administration in vivo comprises systemic administration.

20. The method of claim 19, wherein said transfection facilitating agent comprises a cationic lipid.

21. The method of claim 20, wherein said transfection facilitating agent further comprises cholesterol.

22. The method of claim 21, wherein said cationic lipid contains DOTMA and said DOTMA and said cholesterol are present in a 4:1 molar ratio of DOTMA to cholesterol.

23. The method of claim 19, wherein said nucleic acid molecule expression is increased by sonoporation between 6 and 270 fold relative to expression of said nucleic acid molecule after systemic delivery of said complex to the tumor without sonoporation of the tumor.

24. The method of claim 17, wherein the tumor is sonoporated for between about one and about fifteen minutes.

25. The method of claim 19, wherein the tumor is sonoporated within about five minutes of the systemic administration.

26. The method of claim 19, wherein said a quantity of nucleic acid molecule systemically administered is between about 15 micrograms and 90 micrograms.

27. The method of 18, wherein said immunomodulator is IL-12.

28. The method of claim 17, wherein said transfection facilitating agent comprises a protective, interactive and non-condensing compound.

29. The method of claim 17, wherein said transfection facilitating agent is selected from the group consisting of: one or more polyvinyl-pyrrolidones, one or more cationic lipids, one or more cationic lipids with neutral co-lipids, one or more liposomes, one or more peptides, and one or more lipopeptides.

30. The method of claim 17, wherein said method results in an antibody response.

31. The method of claim 17, wherein said method promotes an immune response.

32. The method of claim 17, wherein the tumor is in a mammal.

33. The method of claim 17, wherein said mammal is a human.

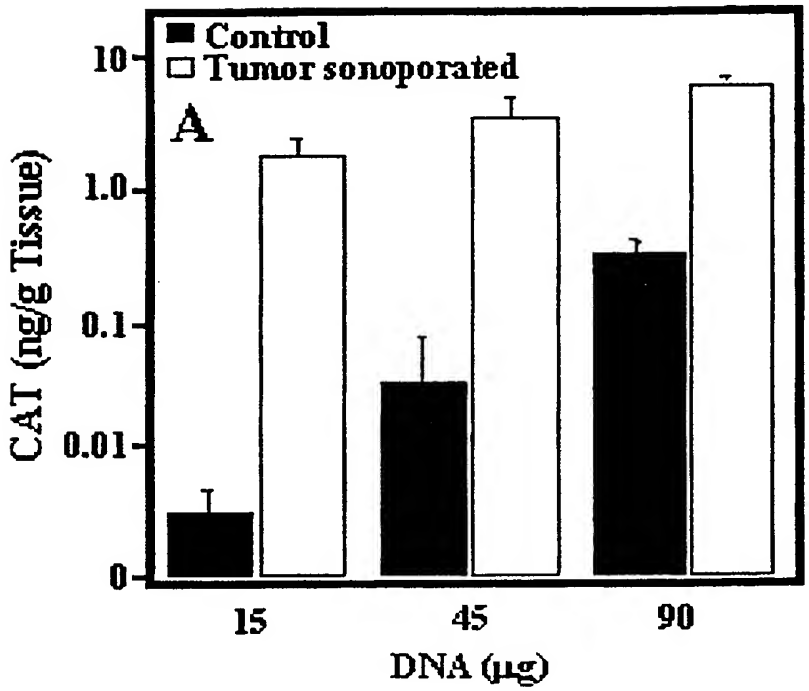
34. The method of claim 17, wherein said nucleic acid molecule encodes a cancer antigen.

35. The method of claim 18, wherein said tumor antigen is MAGE 1, and said tumor is melanoma.

36. A kit for use in a method of any one of claims 17-35 comprising a container for providing a formulation comprising a nucleic acid molecule and a transfection facilitating agent, and either (*i*) a sonoporation device for delivering said formulation to a tumor, said sonoporation device capable of being combined with said container, or (*ii*) a instruction explaining how to deliver said formulation with said sonoporation device.

37. A method for making a kit of claim 36 comprising the steps of combining a container for providing a formulation comprising a nucleic acid and a transfection facilitating agent with either (*i*) a sonoporation device for delivering said formulation to a tumor, wherein said sonoporation device is capable of being combined with said container, or (*ii*) instructions explaining how to deliver said formulation with the sonoporation device.

Figure 1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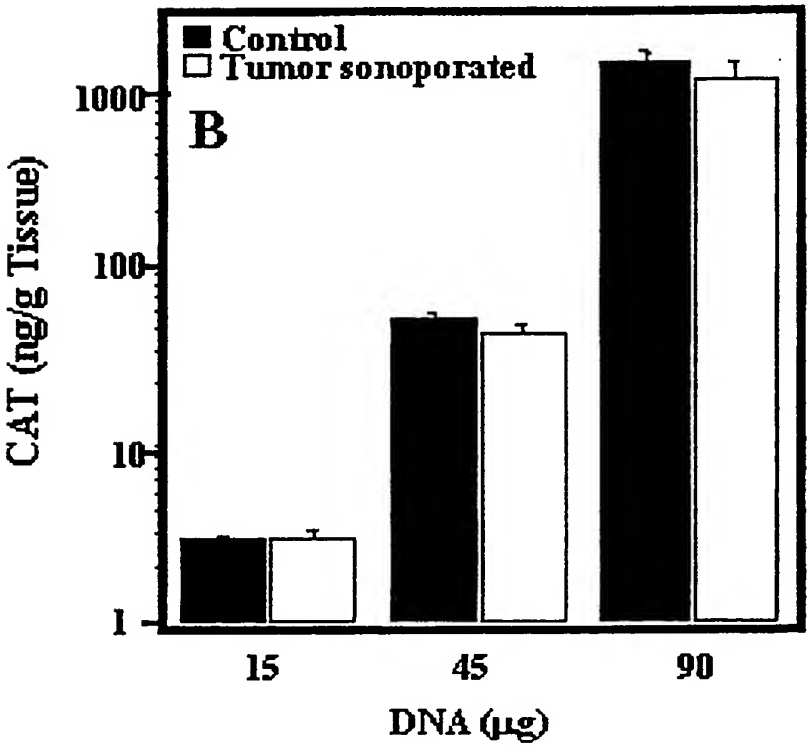
DNA (µg)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10
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15
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DNA (µg)

Figure 2

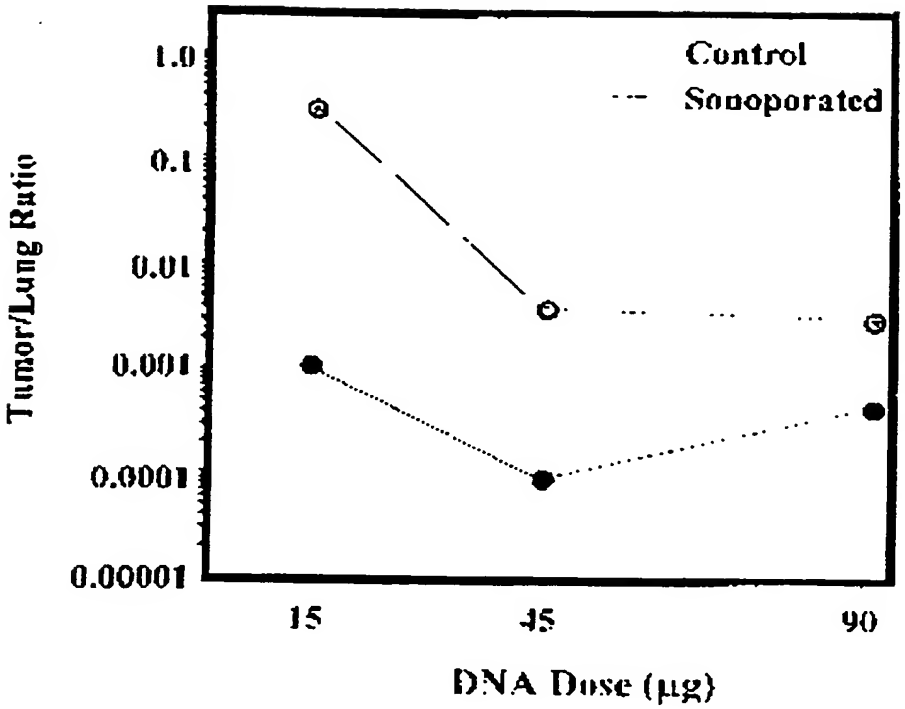


Figure 3

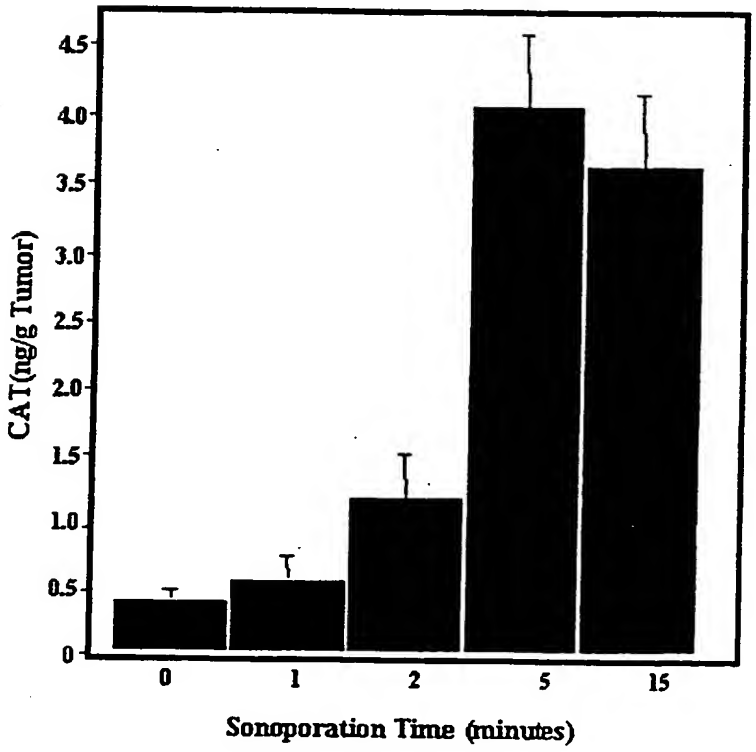


Figure 4

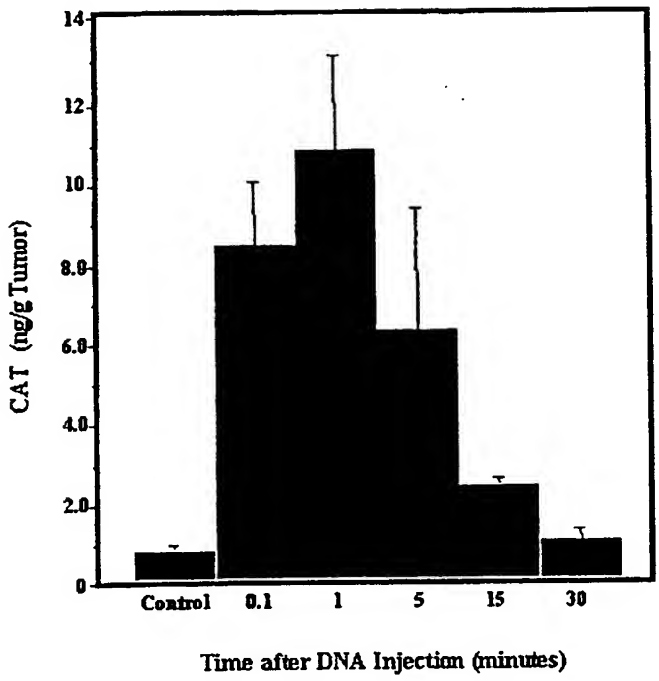


Figure 5

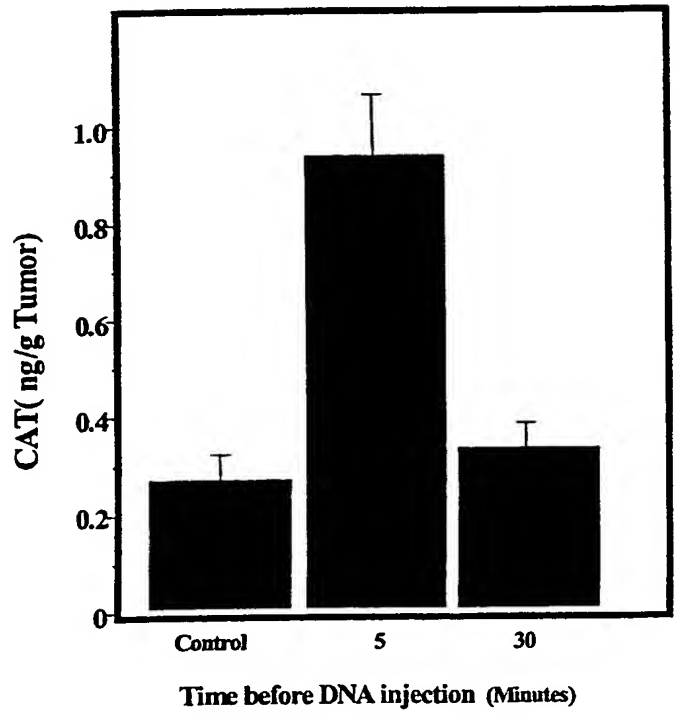


Figure 6

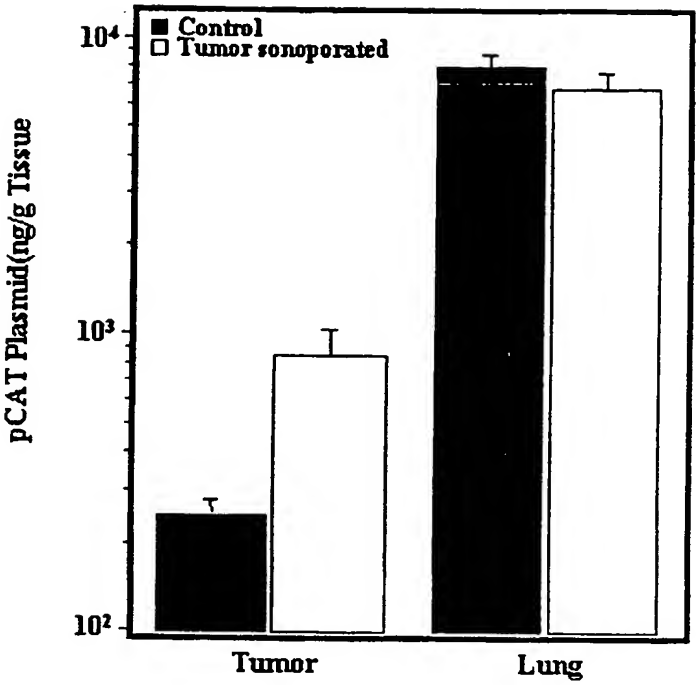


Figure 7

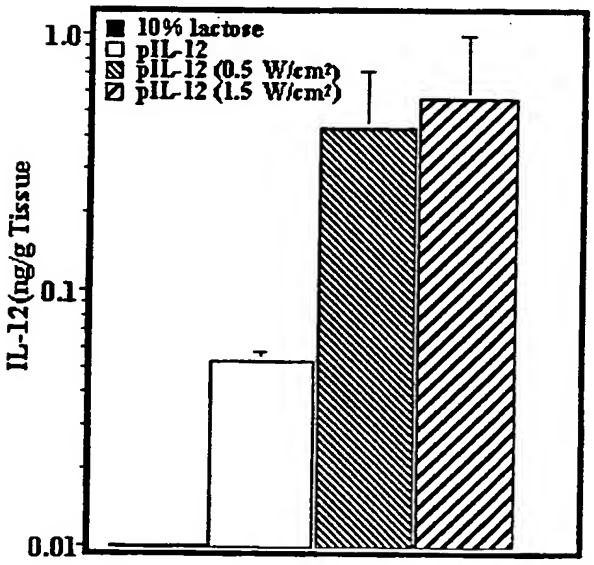
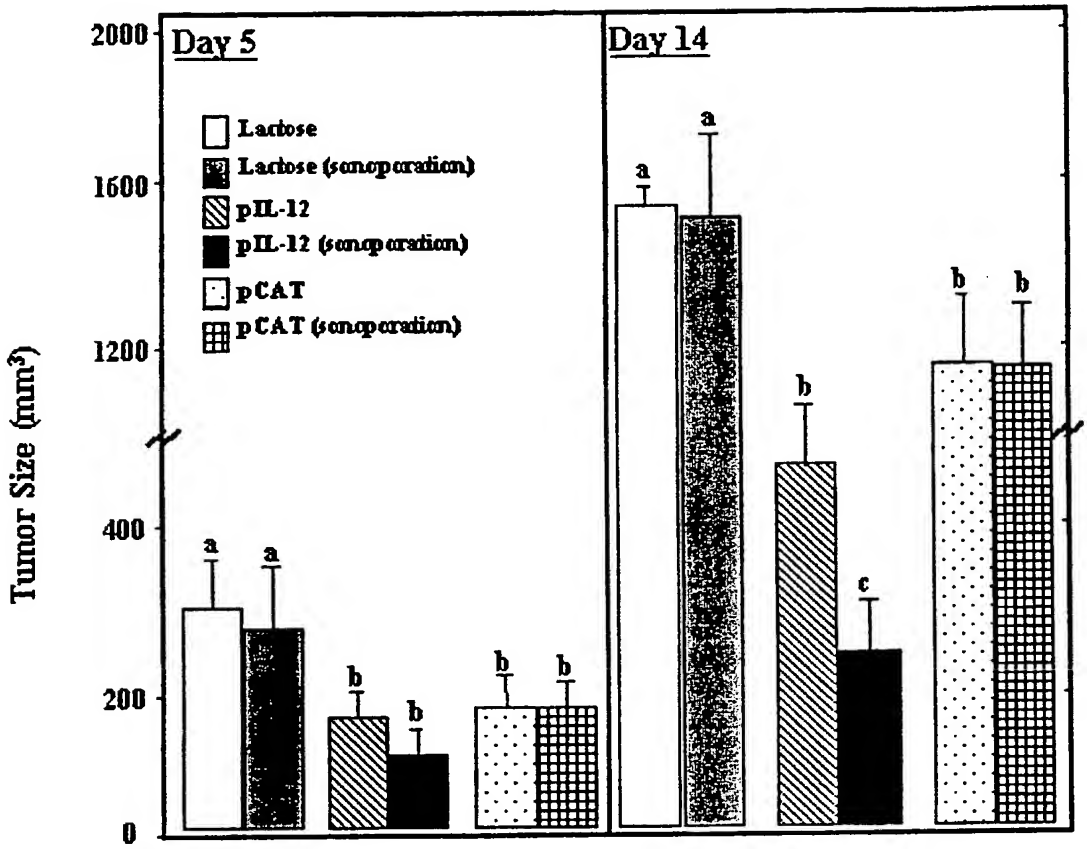


Figure 8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pIL-12 (sonoporation)
pCAT
pCAT (sonoporation)
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1600
1200
400
200
0
a
a
b
b
b
b
a
a
b
c
b
b

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 00/20631

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K48/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal, BIOSIS, CHEM ABS Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 40679 A (IMARX PHARMACEUTICAL CORP) 6 November 1997 (1997-11-06)	1,2,8,9, 13,14, 17,18, 24,28, 29,32, 33,36,37
Y	page 4, line 3 -page 5, line 9 page 12, line 24 -page 13, line 5 page 15, line 14 -page 16, line 2 page 19, line 7 -page 20, line 7 page 25, line 5 -page 26, line 20 page 37, line 23 -page 41, line 2 page 47, line 22 -page 49, line 17 page 50, line 3 - line 16 page 61; example 9 page 67 -page 68; example 17 -/--	7,12,15, 16,27, 30,31, 34,35

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 2000	11/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Sitch W

INTERNATIONAL SEARCH REPORT

Inter)nal Application No
PCT/US 00/20631

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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